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UNIVERSITY OF ALBERTA

**CYTOKINE GENE EXPRESSION IN
IMMUNE-MEDIATED DISEASES**

BY

AMR EL-SHEIKH

A thesis submitted to the faculty of Graduate Studies and Research in
partial fulfillment of the requirement for the degree of Master of
Sciences.

IN

EXPERIMENTAL PATHOLOGY

DEPARTMENT OF PATHOLOGY

EDMONTON, ALBERTA

FALL 1991



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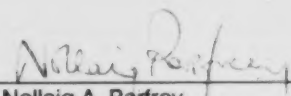
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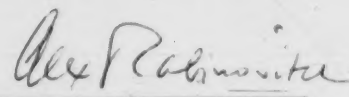
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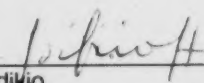
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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Cytokine Gene Expression in Immune-mediated Diseases submitted by Amr El-Sheikh in partial fulfillment of the requirements for the degree of Master of Science in Pathology.


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Dr. H. Idikio

Chairman of committee
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September 24, 1991

DEDICATION

To the searchers for the truth in my family
past, present, and future.

ABSTRACT

Gene expression of the cytokines tumor necrosis factor alpha (TNF α) and Interferon gamma (IFN γ) was studied in minor graft-versus-host-disease (GVHD) and diabetes mellitus type I (IDDM). Using CBA/J, B10.BR, and Balb/c mice, bone marrow transplantation was executed to generate syngeneic controls, minor GVHD, and major GVHD. The liver and tongue of these recipients were harvested on days 7 and 16 following transplantation, and changes of TNF α and IFN γ gene expression were studied by Northern blot analysis. Increased TNF α transcripts were detected in the nonlymphoid target tissues, the liver and tongue, on day 7 during the development of the phase of tissue injury of acute minor GVHD. Increased IFN γ transcripts were not detected.

Using a murine model, the NOD mouse, changes of TNF α gene expression in the pancreas were studied by means of Northern blot analysis, construction and screening of cDNA library, and polymerase chain reaction (PCR) technology. The first two techniques were unable to detect the presence of TNF α message in the pancreas. After amplification using PCR, TNF α message was found to be expressed in freshly diabetic NOD mouse pancreas.

These findings provide evidence to support a role for TNF α in directly contributing to immune-mediated tissue injury in these diseases.

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AMR

TABLE OF CONTENTS

1. **Chapter 1 :** Introduction (p1) :
 - I. Autoimmunity (p2)
 - II. Diabetes mellitus type I (p3)
 - III. The role of HLA.DR in IDDM (p4)
 - IV. The role of autoreactive T-lymphocytes in IDDM (p5)
 - V. Cytokines with cytotoxic properties : (p6)
 - (a) Tumor necrosis factor α (p6)
 - (b) Interferon γ (p8)
 - VI. The role of TNF α and IFN γ in tissue destruction (p10)
 - VII. The role of TNF α and IFN γ in minor minor GVHD and IDDM (p10)
 - VIII. References (p12)

2. **Chapter 2 :** The role of TNF α and IFN γ in minor GVHD (p16) :
 - I. Introduction (p17)
 - II. Materials and methods (p18) :
 1. Animals and disease induction (p18)
 2. Northern analysis (p19)
 - III. Results (p20)
 - IV. Discussion (p31)
 - V. References (p33)

3. **Chapter 3 :** The role of TNF α and IFN γ in diabetes mellitus type I (p36) :
 - I. Introduction (p37)
 - II. Materials and methods (p38) :
 1. Animals and monitoring (p38)
 2. Northern analysis (p39)
 3. Construction and screening of the cDNA library (p40) :
 - a. ZAP cDNA synthesis (p41)
 - b. Ligation of cDNA into vector arms (p41)
 - c. Packaging of the library (p42)
 - d. Plating (p42)
 - e. Amplification of the library (p42)
 - f. Library screening (p.42)
 4. Amplification of cytokine message from the pancreas using the PCR (p43)

III. Results (p44) :

1. Histological examination (p44)
2. Northern analysis (p51)
3. Construction and screening of the cDNA library (p57)
4. Amplification of the cytokine message using PCR (p57)

IV. Discussion (p62)

V. References (p66)

4. Chapter 4 :

Conclusion (p69) :

- I. The results and the hypothesis (p70)
- II. TNF α in minor GVHD.(p70)
- III. TNF α in insulin-dependent diabetes (p70)
- IV. Future directions (p72)
- V. References (p73)

TABLES

1. **Table 2.1 :** Video-densitometric readings of the amount of TNF α and β -actin mRNA in the GVHD samples and their ratio (pp 28,29).
2. **Table 3.1 :** Total RNA extraction from NOD and Balb/c pancreata (p52).
3. **Table 3.2 :** Plasmid DNA and TNF α DNA amounts and band intensities in the PCR experiment (p60).

Figures

1. **Figure 1.1:** Schematic representation of the level of $\text{TNF}\alpha$ and its effects on the tissues (p7).
2. **Figure 1.2:** Schematic representation of the cellular effects of $\text{TNF}\alpha$ (p8)
3. **Figure 1.3:** A summary of $\text{IFN}\gamma$'s targets and its effects on them (p9).
4. **Figure 2.1:** Summary of $\text{TNF}\alpha$ gene expression in the target tissues of the minor GVHD experiment (p30).
5. **Figure 3.1 :** A graph showing the relationship between the starting amount of $\text{TNF}\alpha$ DNA in the PCR reactions and the band intensity on the agarose gel (p61).

PLATES

1. Plate 2.1a : The agarose/formaldehyde gel showing RNA isolated from the liver of some of the recipients in the minor GVHD experiment (p21).
2. Plate 2.1b : The agarose/formaldehyde gel showing RNA isolated from the liver of rest of the recipients in the minor experiment (p22).
3. Plate 2.2 : The agarose/formaldehyde gel showing RNA isolated from the tongue of the recipients in the minor GVHD experiment (p23).
4. Plate 2.3 : TNF α Northern analysis of the liver in the minor GVHD experiment (p24).
5. Plate 2.4 : β -actin Northern analysis of the liver in the minor GVHD experiment (p25).
6. Plate 2.5 : TNF α Northern analysis of the tongue in the minor GVHD experiment (p26).
7. Plate 2.6 : β -actin Northern analysis of the tongue in the

minor GVHD experiment (p27).

8. Plate 3.1 : H and E stain showing a section from NOD#15's pancreas (p45).
9. Plate 3.2 : H and E stain showing a section from NOD#33's pancreas (p46).
10. Plate 3.3 : H and E stain showing a section from NOD#34's pancreas (p47).
11. Plate 3.4a : H and E stain showing a section from NOD#50's pancreas (p48).
12. Plate 3.4b : H and E stain showing a section from NOD#50's pancreas (p49).
13. Plate 3.4c : H and E stain showing a section from NOD#50's pancreas (p50).
14. Plate 3.5 : The agarose/formaldehyde gel showing total RNA extracted from NOD and Balb/c mice pancreata (p54).
15. Plate 3.6 : Northern blot analysis of total RNA from the NOD and Balb/c mice, β -actin is the probe used in this experiment (p55).

16. **Plate 3.7 :** The agarose/formaldehyde gel showing mRNA extracted from NOD pancreas (p56).
17. **Plate 3.8 :** An agarose gel showing the result of the first PCR experiment (p58).
18. **Plate 3.9 :** An agarose gel showing the result of the second PCR experiment (p59).

SYMBOLS AND ABBREVIATIONS

1. APC : antigen presenting cell.
2. CD : cluster designation.
3. cDNA : complementary deoxyribonucleic acid.
4. CHX : cycloheximide.
5. DNA : deoxyribonucleic acid.
6. GVHD : Graft-versus-host disease.
7. HLA : human leukocyte antigen.
8. IDDM : insulin-dependent diabetes mellitus.
9. IFN γ : interferon gamma.
10. IL-1 : interleukin 1.
11. IL-2 : interleukin 2.
12. IL-6 : interleukin 6.
13. LT : lymphotoxin
14. MHC : major histocompatibility complex.
15. mRNA : messenger ribonucleic acid.
16. NK : natural killer.
17. NOD : non-obese diabetic.
18. RFLP : restriction fragment length polymorphism.
19. RNA : ribonucleic acid.
20. PCR : polymerase chain reaction.
21. TNF α : tumor necrosis factor alpha.

Chapter 1

INTRODUCTION

I. Autoimmunity :

Autoimmune diseases occur because the individual's immune system attacks the tissues of the same individual. The occurrence of diseases such as rheumatoid arthritis, systemic lupus erythematosus, and rheumatic fever have been attributed to autoimmunity¹². The generation of an autoimmune disease can be divided into two stages. First, the initiation stage during which development of autoantibodies or autoreactive cells occurs, and second, tissue damage due to immune reactions³.

The ability of the immune system to distinguish self from nonself is not only a marvel but also a riddle that is a central issue challenging our understanding of science and medicine. It appears that a properly functional immune system relies on keeping this process of self-versus-nonself recognition in a delicate balance. If the immune system shifts to recognize self as nonself, autoimmunity develops as in the case of the rheumatoid arthritis.

The recognition process depends on the interaction between two components of the immune system: T-cells and the major histocompatibility complex (MHC) determinants present on the individual's cells. Therefore, autoimmunity and immune anergy result from malfunctioning at this level of organization. The expression of class II MHC determinants (HLA-DR in humans, Ia in animals) is usually very restricted. They are normally present only on macrophages, dendritic cells, vascular endothelial cells, Langerhans cells, activated T-cells, B-cells, and thymic, gastrointestinal and renal epithelial cells³. Aberrant expression of these proteins, as was observed in graft-versus-host disease (GVHD), has many autoimmune manifestations⁶.

T-cell recognition of class II MHC molecules leads to the release of a variety of lymphokines such as interleukin 2 (IL-2), macrophage activating factor (MAF), and interferon gamma (IFN γ). These lymphokines promote the process of immune-mediated damage either directly or by enhancing the expression of class II MHC molecules on the antigen presenting cells (APCs) and through recruitment of other immune cells that eventually result in the destruction of the individual's tissues. However, the exact mechanism that leads to the destruction is not yet fully understood.

In this thesis, diabetes mellitus type I and minor graft-versus-host disease were chosen as models of immune-mediated diseases and the role of cytotoxic lymphokines in the pathogenesis of these diseases was investigated. The relationship was established by correlating changes in gene expression of these lymphokines and the phase of tissue injury in these diseases.

II. DIABETES MELLITUS TYPE I :

Diabetes is one of the most common metabolic disorders in humans¹⁸. However, the basic lesion responsible for the disease is unknown. On the basis of superficial characteristics cases were grouped together. Based on the clinical and epidemiological evidence, insulin-dependent diabetes mellitus (IDDM or type I) has been considered a separate entity from noninsulin-dependent diabetes mellitus.(NIDDM or type II).

It is estimated that one person in three hundred develops insulin-dependent diabetes¹. The onset of this disease usually occurs in early adulthood. IDDM is usually characterized pathologically by a massive lymphocyte infiltration in and around the islets of Langerhans in the pancreas (insulitis) and the appearance of islet and islet surface antibodies. IDDM is an autoimmune disease²⁰. The disease is attributable to the insulitis and the selective and total absence of the pancreatic β -cells which results in insulinopenia.

III. THE ROLE OF HLA.DR IN IDDM :

After the cellular and serological recognition of class II molecules became available, a major breakthrough in the study of autoimmunity has occurred. In 1981, it became evident that the HLA.DR3 and HLA.DR4 were highly associated with diabetes¹³. The incidence of the presence of DR3 and/or DR4 markers in Caucasoid individuals with IDDM was approximately 95%. On the other hand HLA.DR2 was found to be a protective marker from IDDM¹⁷.

As techniques in molecular biology were more modernized, the restriction fragment length polymorphism (RFLP) technique was able to show stronger correlation between the HLA-DQ beta fragment pattern and IDDM¹⁵. Thus, the susceptibility for diabetes has been progressively moving toward the centromere of human chromosome 6¹¹. A mutation at amino acid residue #57 of the β -chain renders the individual susceptible to diabetes. A mutation at this position is not sufficient to bring about IDDM⁸. However, a mutation of the wild type charged aspartic residue to a noncharged one, like valine or serine, in combination with polymorphic DQ

α -chains⁹ can lead to the development of diabetes. The details of the interactions between these chains are not fully clear yet, but it seems like a mutation such as that of position 57 can, in combination with some mutation in the α -chains, change the three-dimensional configuration of the MHC molecule in such a way that can result in attracting autoreactive T-cells.

IV. THE ROLE OF AUTOREACTIVE T-LYMPHOCYTES IN IDDM :

The second component of the immune system that is involved in development of autoimmunity is the T-cells. In IDDM, the infiltrate that is found in and around the pancreatic islets of Langerhans was examined to determine the type of T-cells involved. Using monoclonal antibodies that phenotypically recognize various compartments of the immune system and identify HLA molecules, a fresh pancreas from a twelve year old child with newly diagnosed diabetes was examined⁴. The majority of the infiltrating lymphocytes were found to be from the cytotoxic/suppressor subpopulation but helper T-cells and natural killer cells were also present in small numbers.

In addition to the presence of cytotoxic T-cells, a marked increase of Class I (HLA.A, B, and C) molecule expression was observed in the affected islet cells. This suggested an active role of these cytotoxic T-lymphocytes in damaging the β -cells directly. These cells are known to produce various cytokines that, in addition to their role in propagating the inflammatory process, are capable of causing direct tissue injury.

V. CYTOKINES WITH CYTOTOXIC PROPERTIES :

Cytokines⁷ are any nonenzymatic peptide mediator of immune-inflammatory responses. In addition, they have multiple hormone-like actions that extend beyond the immune system. The term cytokines now includes lymphokines, monokines, and interferons². They have a primary structure of about several hundred amino acids in length and are the products of single genes. Some of these cytokines such as tumor necrosis factor alpha (TNF α) have a strong cytotoxic capacity.

(a) Tumor Necrosis Factor α :

Tumor necrosis factor (cachectin) is involved in the pathogenesis of infection, inflammation, and tissue remodelling²⁰. TNF α is produced in the body in response to infection or injury. The amount of TNF α produced dictates the action carried out by the cytokine. At low doses, the effect of TNF α seems to be mediating tissue remodelling and inflammation. High doses, on the other hand, lead to tissue injury and shock (figure 1.1).

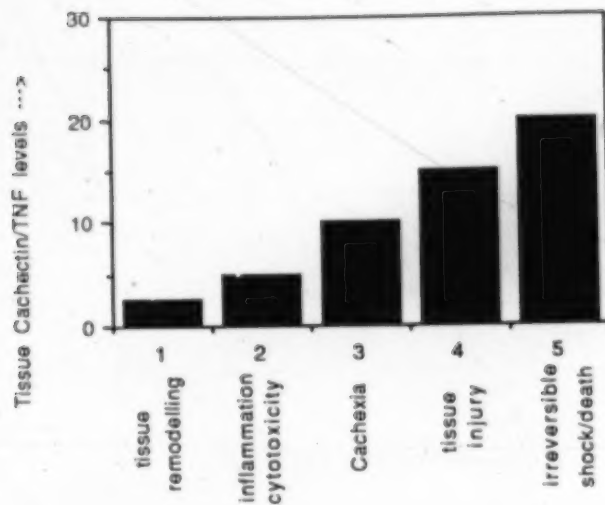


Figure 1.1: Schematic representation of the level of $\text{TNF}\alpha$ and its effects on the tissues.

$\text{TNF}\alpha$ is synthesized and produced by various activated cells. Macrophages are known to be the major producers but T-lymphocytes, natural killer(NK) cells, astrocytes and microglial cells of the brain as well as Kupffer cells of the liver also produce it. $\text{TNF}\alpha$ has multiple cellular effects and figure 1.2 shows a simplified diagram with those effects. $\text{TNF}\alpha$ has been implicated as an active agent in the development of autoimmune diseases like rheumatoid arthritis, and in transplant rejection. Furthermore, it was proved to play an important role in major GVHD¹⁰

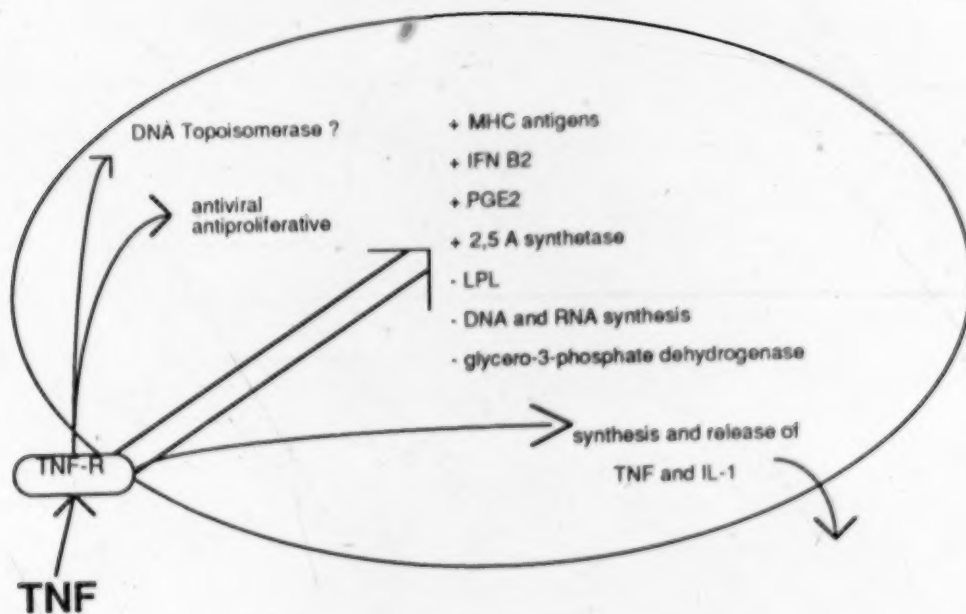


Figure 1.2: Schematic representation of the cellular effects of TNFα (+ means increase, - means decrease of activity, and R is receptor)

(b) Interferon γ :

Interferon- γ (IFN γ) is a small cytokine of about 146 amino acid residues produced mainly by activated T-lymphocytes¹⁹. Depending on the origin of the stimulus, both helper and cytotoxic/suppressor cells may produce IFN γ . In addition, NK cells are also potent producers of the cytokine when appropriately stimulated with IL-1. However, IFN γ is considered the central mediator of the immune system because of the variety of target tissues it has and its multiple effects. Figure 1.3 summarizes those targets and effects.

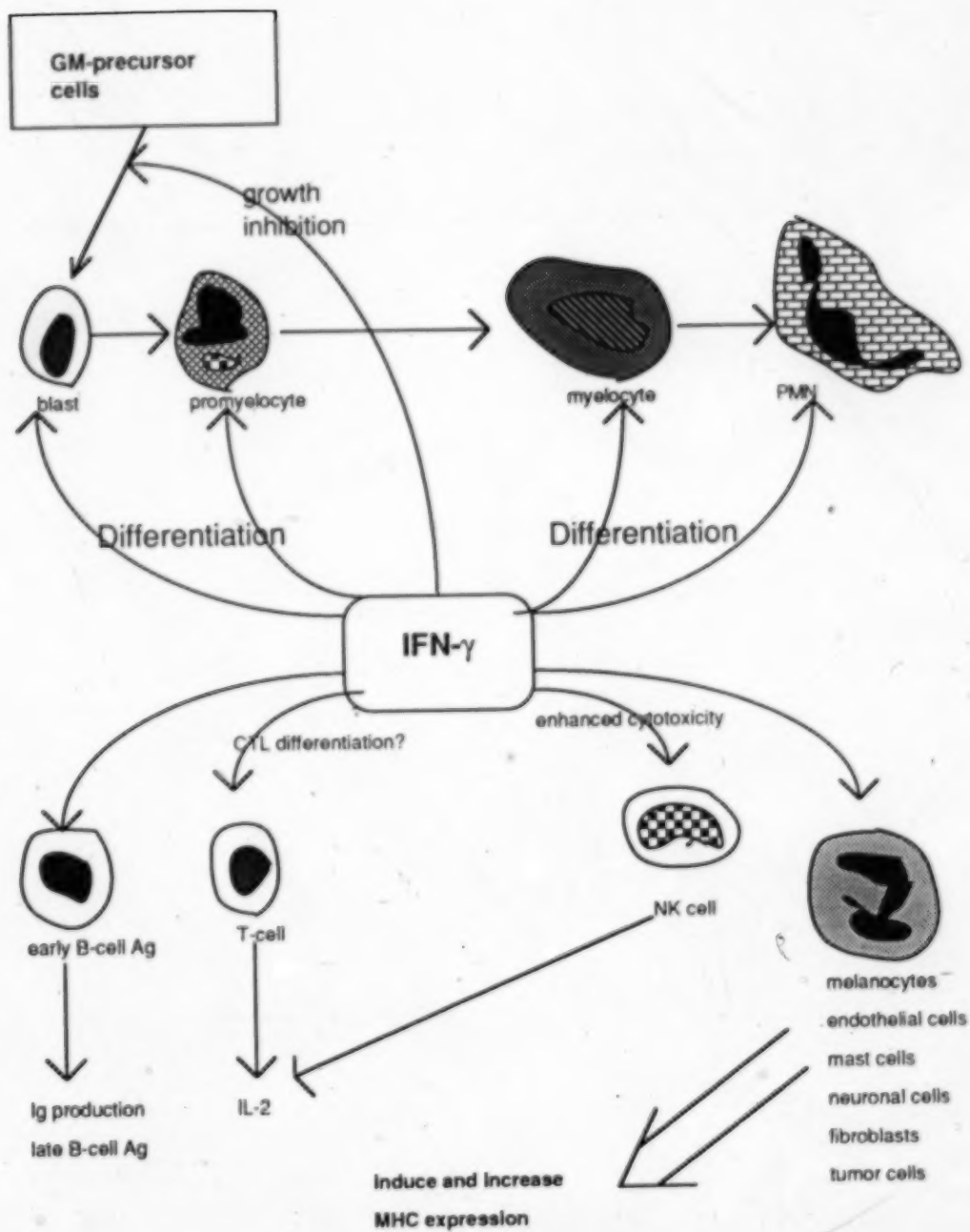


Figure 1.3: A summary of IFN γ 's targets and its effects on them.

IFN γ functions as a major inducer of class I and class II MHC expression¹⁶. More importantly, it is known for its growth inhibitory ability and its strong synergistic effect with other cytokines like TNF α . Furthermore, IFN γ is also known for its direct cytolytic activity against number of tumor cells as well as against normal cells²².

VI. ROLE OF TNF α AND IFN γ IN TISSUE DESTRUCTION :

The role of TNF α and IFN γ was strengthened in acute major GVHD where genes of both cytokines were shown to be overexpressed in the target tissues^{10,23}. Furthermore, TNF α and IFN γ have been shown to have synergistic and cytotoxic effect on rat pancreatic islet cells in monolayer culture¹⁴. This led us to believe that these cytokines may play a role in direct tissue injury during the development of IDDM and minor GVHD.

VII. THE ROLE OF TNF α AND IFN γ IN MINOR GVHD AND IDDM :

Based on the previous finding mentioned above, we used murine models to study *in vivo* the increased expression of both TNF α and IFN γ genes. In the minor GVHD experiment, we used CBA and Balb/c mice (as explained in chapter 2), since they were used in the experiment for major GVHD.

In order to study IDDM, the NOD mouse was chosen as a model. The NOD mouse displays the disease in a very similar manner to that of human. While it is extremely difficult to obtain human tissues at the proper time for studying the immunological processes involved, it is relatively easy to

maintain and manipulate these mice. In the next two chapters, a detailed discussion of these experiments is presented.

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Chapter 2

**THE ROLE OF $\text{TNF}\alpha$ AND $\text{IFN}\gamma$ IN
MINOR GVHD**

I. INTRODUCTION

Graft-versus-host-disease (GVHD) is a systemic disease that frequently complicates bone marrow transplantation and rarely solid organ transplantation. GVHD arises when immunocompetent cells are transferred into a histoincompatible immunosuppressed host. The severity of the disease depends on the extent of histoincompatibility. Clinically, the skin, liver, and gut are preferentially affected.

The tissue damage that occurs in the target organs is dependant on the introduction of the allogeneic T-lymphocytes. However, the mechanisms that are responsible for the destruction of these tissues are not clearly understood. There may be more than one mechanism involved. A direct mechanism involving interaction between the donor T-lymphocytes and the host cells could be responsible for the acute GVHD¹³. Tissue injury may be mediated indirectly by cytokines released by the donor T-cells which leads to the recruitment of other cells to the site of inflammation and consequently release of more cytokines that may contribute to damaging the tissue the host tissues⁹ This study examines the role of $\text{TNF}\alpha$ and $\text{IFN}\gamma$ in the pathogenesis of acute minor GVHD.

II. MATERIALS AND METHODS

1. ANIMALS AND DISEASE INDUCTION :

The tissues of this study were provided by Dr. J. Ferrera (Dana Farber Cancer Centre, Boston)

Eight-to-twelve week old CBA/J(H-2^K) mice were irradiated with a Cs¹³⁷ source at 1000 Rads. They were used as recipients and reconstituted on day 0 with 4×10^6 bone marrow cells and 2×10^6 spleen cells from three groups of donors: CBA/J(H-2^k), B10.BR(H-2^k), and Balb/c(H-2^d). These types of transplantations gave rise to syngeneic controls, minor allogeneic GVHD, and major allogeneic GVHD respectively. Some irradiated CBA/J mice received T-cell-depleted bone marrow transplants (BMT) from CBA/J, B10.BR, and Balb/c mice as further negative controls. In each transplantation type mentioned three recipients were obtained.

All mice were maintained in a laminar air flow hood and their drinking water was acidified. The recipients were sacrificed on days 7 and 16. Half of the mice in each group were injected intraperitoneally with cycloheximide (CHX) at a dose of 200 mg/kg six hours before the sacrifice. This dose and time were found to be optimal for delaying cytokine mRNA degradation *in vivo* ⁴. The tongue and liver were harvested from the three recipients in each group for Northern analysis and rapidly frozen in liquid nitrogen and stored at -70.

2. NORTHERN ANALYSIS :

The tissues from each group were pooled together and total RNA was isolated using the AGPC (Acid Guanidinium Thiocyanate-Phenol-Chloroform) method². 25 µg of the total RNA from each sample was run in each lane of a 1.5% agarose/2.2 M formaldehyde gel along with TNFα positive RNA isolated from the macrophage cell line P388D1¹⁷. The RNA was then transferred to Amersham Hybond-C nitrocellulose filters. The RNA was consequently fixed onto the filters by baking at 80°C under vacuum for two hours.

The filters were then probed with a 446 base pair(bp) fragment of murine TNFα, a 270 bp fragment of IFNγ, and a 2.0 kb Pst I fragment of β-actin³ (from embryonic chick brain) cDNA probes. The murine TNFα fragment was generated using the polymerase chain reaction(PCR) technology¹¹. The primers used in this generation were synthetic 23-mer oligonucleotides corresponding to bps 144-167 and complimentary to bps 567-590 of the published murine TNFα sequence¹². The murine IFNγ was provided by Genentech. The β-actin cDNA probe was used as a positive control to check the quality of the RNA isolated since actin is a housekeeping gene that is expressed in virtually all the cells. The cDNA probes were labeled with ³²P by extension of hexamer random primers⁷. The specific radioactivity of the probes were 4.6×10^8 , 1.4×10^8 , and 4.25×10^8 cpm/µg of DNA template respectively. The filters were washed three times with 2X SSC and 0.1 % SDS solution at room temperature for five minutes each time, then twice with 0.2X SSC and 0.1% SDS solution for 20 minutes each time at 65°C. After drying briefly, the filters were exposed to Kodak X-AR film. Positive blots showed bands of expected size.

Steady state levels of specific mRNA were quantified by video-densitometry of the autoradiograms. Each positive band detected on the autoradiograms was represented by a peak, and the area under the peak corresponded to the intensity of the band. The ratio of TNF α mRNA over β -actin RNA in each sample was calculated.

III. RESULTS

Plates 2.1(a and b) and 2.2 show the ethidium bromide (EtBr) stained agarose/formaldehyde gels of the liver, and tongue RNA samples respectively showing the ribosomal bands of the well preserved RNA. Plates 2.3 and 2.5 show the Northern analyses of the liver and tongue blots respectively probed with TNF α , and plates 2.4 and 2.6 liver and tongue probed with β -actin.

12 11 10 9 8 7 6 5 4 3 2 1



Plate 2.1a : The agarose/formaldehyde gel showing RNA isolated from the liver of some of the recipients in the minor GVHD experiment:

- Lane 1 : TNF α positive RNA (+ve control)
- Lane 2 : ribosomal RNA (marker)
- Lane 3 : d7 syngeneic controls
- Lane 4 : d7 minor GVHD recipients
- Lane 5 : d7 major GVHD recipients
- Lane 6 : d7 syngeneic controls+CHX
- Lane 7 : d7 minor GVHD recipients+CHX
- Lane 8 : d7 major GVHD recipients+CHX
- Lane 9 : d16 syngeneic controls
- Lane 10 : d16 minor GVHD recipients+CHX
- Lane 11 : d16 major GVHD recipients+CHX
- Lane 12 : IFN γ positive RNA

109 8 7 6 5 4 3 2 1

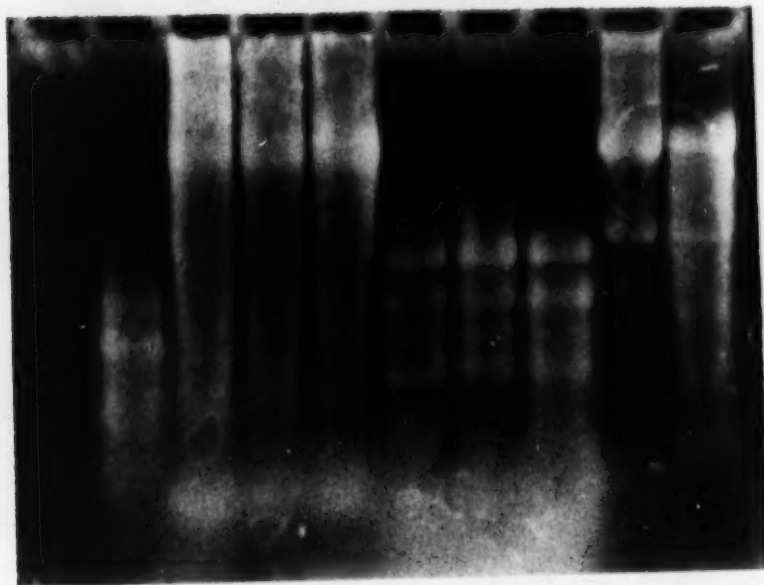


Plate 2.1b : The agarose/formaldehyde gel showing RNA isolated from the liver of the rest of the recipients in the minor GVHD experiment:

- Lane 1: ribosomal RNA (marker)
- Lane 2 : $\text{TNF}\alpha$ positive RNA (+ve control)
- Lane 3 : d7 syngeneic T-cell-depleted bone marrow transplant
- Lane 4 : d7 minor GVHD T-cell-depleted bone marrow transplant
- Lane 5 : major GVHD T-cell-depleted bone marrow transplant
- Lane 9 : $\text{IFN}\gamma$ positive RNA.
- Lane 10: 1.77-0.16 kb RNA ladder (marker)

16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

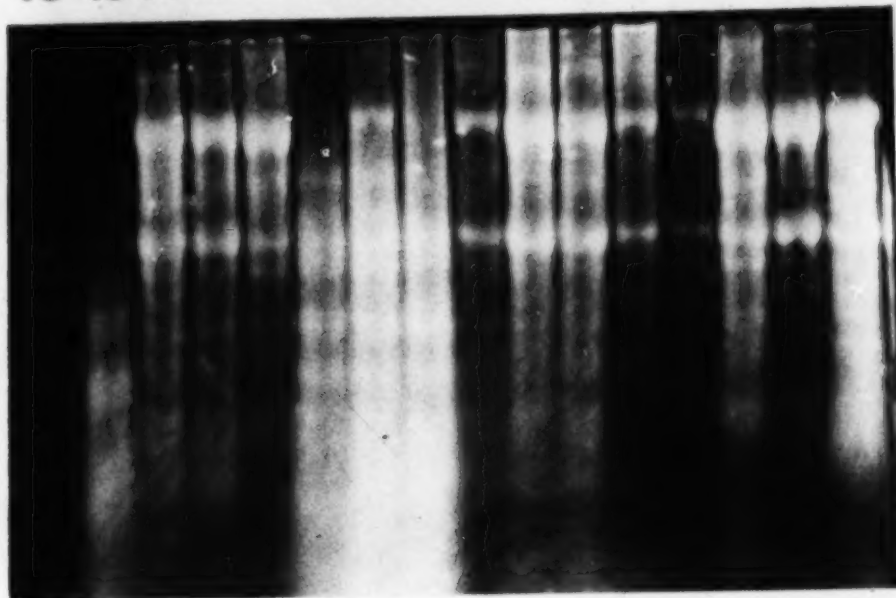


Plate 2.2 : The agarose/formaldehyde gel showing RNA isolated from the tongue of the recipients in the minor GVHD experiment:

- Lane 1: ribosomal RNA (marker)
- Lane 2: TNF α positive RNA (+ve control)
- Lane 3: d7 syngeneic controls
- Lane 4: d7 minor GVHD recipients
- Lane 5: d7 major GVHD recipients
- Lane 6: d7 syngeneic controls+CHX
- Lane 7: d7 minor GVHD recipients+CHX
- Lane 8: d7 major GVHD recipients+CHX
- Lane 9 : d7syngeneic T-cell-depleted bone marrow transplant
- Lane 10: d7minor GVHD T-cell-depleted bone marrow transplant
- Lane 11: d7major GVHD T-cell-depleted bone marrow transplant
- Lane 12: d16 syngeneic controls
- Lane 13: d16 minor GVHD recipients+CHX
- Lane 14: d16 major GVHD recipients+CHX
- Lane 15: IFN γ positive RNA
- Lane 16: 1.77-0.16 kb RNA ladder (marker)

Northern analysis showed an increase in TNF α transcripts in the liver and tongue of the recipients of minor allogeneic transplant (i.e. from B10.BR animals into CBA/J). The transcripts were detected in RNA from day7-cycloheximide treated animals. TNF α expression was not detected in any non-cycloheximide, day16, or T-cell depleted bone-marrow-transplanted animals.

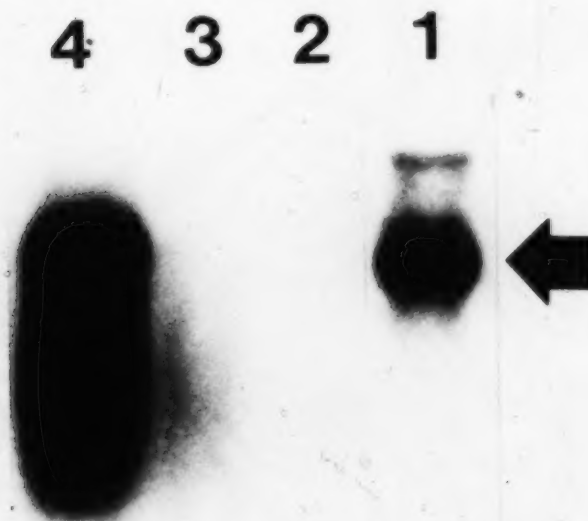


Plate 2.3 : TNF α Northern analysis of the liver in the minor GVHD experiment (arrow showing the TNF α band of 1.77 kb) :

- Lane 1: TNF α positive RNA (+ve control)
- Lane 2: d7 syngeneic controls+CHX
- Lane 3: d7 minor GVHD recipients+CHX
- Lane 4: d7 major GVHD recipients+CHX

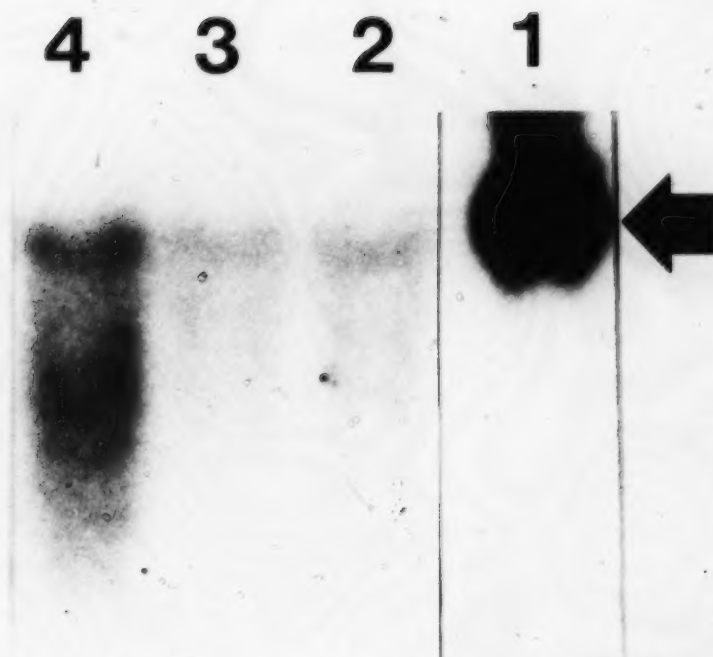


Plate 2.4 : β -actin Northern analysis of the liver in the minor GVHD experiment (arrow showing the β -actin band of 2.0 kb) :

- Lane 1: $\text{TNF}\alpha$ positive RNA (+ve control)
- Lane 2: d7 syngeneic controls+CHX
- Lane 3: d7 minor GVHD recipients+CHX
- Lane 4: d7 major GVHD recipients+CHX

4 3 2 1

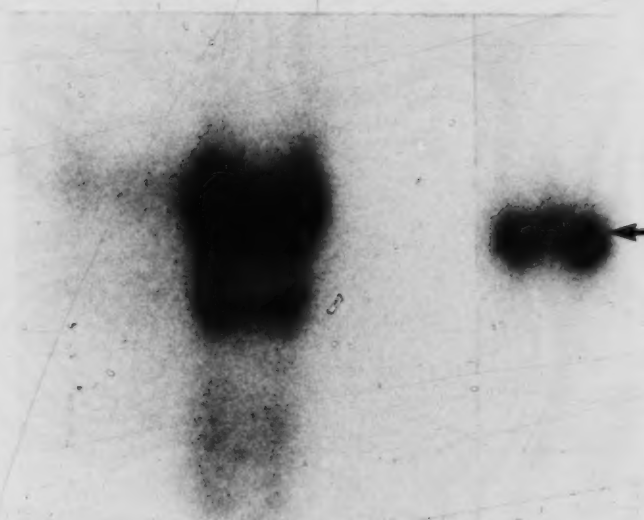


Plate 2.5 : TNF α Northern analysis of the tongue in the minor GVHD experiment (arrow showing the TNF α band of 1.77 kb) :

- Lane 1: TNF α positive RNA (+ve control)
- Lane 2: d7 syngeneic controls+CHX
- Lane 3: d7 minor GVHD recipients+CHX
- Lane 4: d7 major GVHD recipients+CHX



Figure 2.6 : β -actin Northern analysis of the tongue in the minor GVHD experiment (arrow showing the β -actin band of 2.0 kb) :

- Lane 1: TNF α positive RNA (+ve control)
- Lane 2: d7 syngeneic controls+CHX
- Lane 3: d7 minor GVHD recipients+CHX
- Lane 4: d7 major GVHD recipients+CHX

Table 2.1 shows the results obtained from the video-densitometry from the autoradiograms of the liver and tongue of the minor GVHD recipients. The numbers in table 2.1 are the areas of the peaks corresponding to the intensities of the positive bands.

Table 2.1

Video-densitometric readings* of the amount of $\text{TNF}\alpha$ and β -actin mRNA detected in the GVHD samples and their ratio:

Tissue	Sample	$\text{TNF}\alpha$ (a)	β -actin (b)	Ratio (a/b)
Liver	d7 syngeneic	0.00	2.43	0.00
	d7 minorGVHD	0.00	2.25	0.00
	d7 majorGVHD	0.00	3.27	0.00
	d7 syngeneic+CHX	0.00	2.00	0.00
	d7 minorGVHD+CHX	0.10	2.20	0.04
	d7 majorGVHD+CHX	4.46	3.26	1.36
	d16 syngeneic	0.00	3.10	0.00
	d16 minorGVHD	0.00	1.60	0.00
	d16 majorGVHD	0.00	2.03	0.00
	BMT from CBA/J	0.00	2.30	0.00
	BMT from B10.BR	0.00	1.70	0.00
	BMT from Balb/c	0.00	3.23	0.00
	$\text{TNF}\alpha$ positive control (P388D.1 cell line RNA)	4.23	4.56	0.93

Table 2.1 cont'd

Tissue	Sample	TNF α	β -actin	Ratio
		(a)	(b)	(a/b)
Tongue	d7 syngeneic	0.00	2.73	0.00
	d7 minorGVHD	0.00	2.85	0.00
	d7 majorGVHD	0.00	3.64	0.00
	d7 syngeneic+CHX	0.02	3.55	0.01
	d7 minorGVHD+CHX	2.90	3.80	0.76
	d7 majorGVHD+CHX	0.05	1.40	0.04
	d16 syngeneic	0.00	2.42	0.00
	d16 minorGVHD	0.00	1.95	0.00
	d16 majorGVHD	0.00	2.81	0.00
	BMT from CBA/J	0.00	3.23	0.00
	BMT from B10.BR	0.00	2.98	0.00
	BMT from Balb/c	0.00	2.85	0.00
	TNF α positive control (P388D.1 cell line RNA)	1.27	4.14	0.31

* These figures represent the areas of the peaks obtained from each band

The amount of TNF α transcript found in the liver of minor GVHD is about 0.032 of that detected in major GVHD (table 2.1) and about 0.047 of the TNF α positive RNA used as a control in that particular experiment.

In the tongue, the amount of transcript found in the minor GVHD recipients was almost twenty times as much that detected in the major GVHD counterparts. In addition, the amount of transcript in the minor GVHD recipients was 2.45 times of that found in the positive control used in that experiment. The following histogram (figure 2.1) summarizes the results obtained from the minor GVHD experiment:

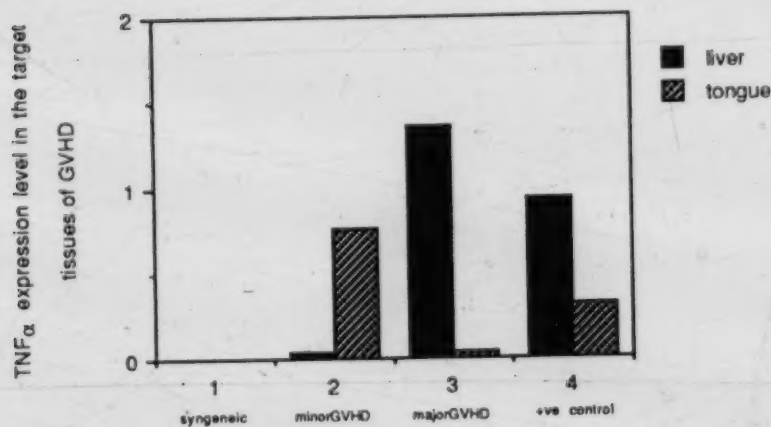


Figure 2.1: Summary of TNF α gene expression in the target tissues of the minor GVHD experiment.

IV. DISCUSSION

TNF α is a pleiotropic cytokine that is produced by a wide variety of cells types. TNF α plays a role in tissue remodelling at low levels whereas at high doses it leads to inflammation and direct tissue injury¹⁵.

TNF α is a good candidate for causing such direct injury. It is produced by a wide variety of cells including macrophages⁸, lymphocytes⁵, natural killers⁶, and mast cells¹⁶, which all could be at the site of inflammation. TNF α was shown to be required for the development of acute GVHD¹⁴. It was shown that anti-TNF α antibody entirely prevented the cutaneous and intestinal lesions of the acute phase of GVHD as well as reducing the overall mortality. Work in our laboratory has shown markedly increased TNF α transcripts in the target tissues of acute allogeneic GVHD during the phase of tissue injury¹¹. Our laboratory has also shown increased IFN γ at the same time in those tissues¹⁰. Those studies were performed in mice transplanted across complete MHC differences as well as non-MHC differences. It was unknown whether the immune mechanisms leading to acute minor GVHD are the same as those leading to major GVHD. In this study, we explored the possibility that TNF α and IFN γ may also be involved in the pathogenesis of minor GVHD.

The two target organs chosen in this experiment showed two different profiles for the increase of the TNF α message. In the liver, a small amount of transcript, in comparison with the major GVHD was found in the minor GVHD recipients. In addition, no transcript was found in the syngeneic recipients. In the tongue, the profile of TNF α increase was different. There was more transcript found in the minor GVHD recipients than in the syngeneics, the TNF α positive RNA, and even the major GVHD

ones. This was an unexpected finding as the tongue is extensively involved by day 7 during major GVHD¹¹.

The fact that the TNF α message was detected only in day 7 and not day 16 indicate by that time the damage has already occurred and the immunological processes that elicited the damage have disappeared. Furthermore, it appears that the cycloheximide treatment was necessary in this experiment in order to guarantee the presence of some cytokine mRNA. The role of cycloheximide was shown to be the delay of degradation of the cytokine messages⁴.

The results from this experiment show that TNF α gene is hyperexpressed during the phase of tissue injury in minor GVHD. This was shown by comparison of the minor GVHD animals to the syngeneic controls. Furthermore, the profile of hyperexpression in the nonlymphoid target organs differs from that observed in major GVHD. This was shown by comparison of the minor GVHD animals to the major GVHD ones. From these findings and the knowledge of TNF α as a cytolytic molecule, we can conclude that its presence in the target tissues provides evidence to support the possible role of TNF α in the progression of minor GVHD

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Chapter 3

**THE ROLE OF $\text{TNF}\alpha$ AND $\text{IFN}\gamma$ IN
DIABETES MELLITUS TYPE I**

I. INTRODUCTION

Diabetes mellitus type I results from autoimmune destruction of the β -cells in the islets of Langerhans of the pancreas. The destruction of β -cells is preceded and accompanied by a mononuclear infiltrate of the islets of Langerhans (insulitis). The majority of these cells are reported to be activated T-lymphocytes, predominantly CD8⁺ cytotoxic/suppressor T-cells².

In addition to the T-lymphocytes, cells of the B-lymphocyte lineage were also observed with a predominance of those containing cytoplasmic IgG¹². The presence of both types of lymphocytes in the target region suggested that there might be more than one mechanism involved in the process leading to the destruction of the β -cells. This study was only concerned with the mechanism involving the T-lymphocytes, or the cell-mediated pathway.

The NOD mouse is an excellent model of the human disease. The NOD mouse displays a clinical picture very similar to that in human¹⁴. The onset of overt diabetes in the NOD mouse is abrupt, and preceded, as well as accompanied, by lymphocyte infiltration in and around the pancreatic islets. As early as four to six weeks of age, the infiltration of B and T-lymphocytes in and around the islets is observed. By the age of thirty weeks, 72% of female and 39% of male mice develop full-blown diabetes (Parham, 1990). It is interesting to note that this sex difference is not observed in humans. The transfer of the disease requires both CD4⁺CD8⁻ and CD4⁻CD8⁺ lymphocytes¹⁵, suggesting that the disease is both T-cell dependent and T-cell mediated.

This study examines the potential contribution of TNF α and IFN γ in leading to pancreatic β -cell injury during spontaneous diabetogenesis in the NOD mouse.

II. MATERIALS AND METHODS

1. Animals and Monitoring :

Twelve week old female NOD mice were housed in a laminar flow hood and their water was acidified. Their urine was monitored twice weekly for glucose intolerance using Tes-tape (a glucose enzymatic test strip that turns from yellow to green if the glucose concentration in urine is more than 1/10%, or roughly 200 mg/dl). If found diabetic, the animal was sacrificed within 48 hours from the test. The pancreas was harvested and a small piece was fixed in 10% formalin and embedded in parafin. 5 μ section were stained with hematoxylin and eosin (H and E), and examined under light microscopy. The rest of the pancreas was immediately either thrown in 4M Guanidium solution or frozen in liquid nitrogen to prevent RNA degradation. Some freshly diabetic NOD mice were injected with cycloheximide at a dose of 200 mg/kg six hours before sacrifice in order to delay cytokine mRNA degradation⁵. In addition, pancreata from Balb/c mice were harvested to be used as a negative control.

2. Northern Analysis :

Total RNA extraction⁴ was carried out on pancreata from seven freshly diabetic NOD mice (four of which were treated with cycloheximide six hours before the sacrifice) and from three Balb/c female mice. 90 µg of total RNA was run in each lane of a 1.5% agarose/ 2.2 M formaldehyde gel along with two RNA marker lanes (9.5-0.24 and 1.77-0.16 kb). The markers were purchased from BRL, and 4 µg of each was loaded on the gel. The RNA from the gel was transferred later to Amersham hybond-C nitrocellulose filters and baked at 80°C under vacuum for two hours. The filters were then probed with TNFα (PCR generated in our lab- please refer to the minor GVHD experiment in the previous chapter) and IFNγ (from Genentech) cDNA probe labeled with ³²P (dCTP) by extension of hexamer random primers⁶. The specific radioactivity of the probes were measured to be 1.3×10^9 , and 1.8×10^9 cpm/µg of DNA respectively. The blots were also probed with a control probe, β-actin cDNA. This was 2.0 bp Pst I fragment of embryonic chick brain β-actin cDNA. The filters were washed twice with 2X SCC and 0.1% SDS for 10 minutes each at room temperature. Then they were washed once with 0.2X SCC and 0.1% SDS for 30 minutes at 65°C. After, drying briefly, the filters were exposed to Kodak X-AR film.

In order to increase the sensitivity of the Northern analysis, mRNA was extracted using the Promega kit, PolyA TractTM mRNA Isolation System from total RNA extracted from three freshly diabetic NOD mice. In addition, mRNA was extracted from the remainder of the total RNA previously extracted from the cycloheximide-treated mice. The mRNA samples were run on an agarose/formaldehyde gel along with TNFα positive RNA (total RNA extracted from the macrophage lymphoma cell line, P388D.1¹⁶ from the NOD pancreatic total RNA, and Balb/c pancreatic

total RNA. The gel was transferred as discussed above. Furthermore, two radioactive nucleotides (dCTP and dATP) were used for labeling the probes which resulted in specific radioactivities of 4.6×10^9 and 5.1×10^9 cpm/ μ g of DNA for TNF α and INF γ cDNA probes respectively.

3. Construction and Screening of the cDNA Library:

A freshly diabetic NOD mouse (NOD#50) was sacrificed for this experiment and the pancreas was harvested. The pancreatic mRNA was extracted using the Invitrogen kit, FastTrack. The diabetic pancreas was homogenized by passing it through 18 gauge needle in the lysis buffer (provided by the kit) and incubated at 45°C in slow shaking water bath for 30 minutes. The homogenate was centrifuged at 4,000x g for 5 minutes and the supernatant was transferred to a new tube. The concentration of NaCl was adjusted to 0.5 M and one tablet of oligo(dT) was added. After the tablet dissolved by gentle shaking, the tube was rocked gently for 30 minutes at room temperature and pelleted again by centrifugation. The pellet was resuspended in 20 ml of binding buffer and again pelleted then resuspended in 10 ml of the binding buffer. The oligo(dT) was pelleted and washed three times in low salt wash and finally resuspended in 0.8 ml of the washing buffer and pipetted through the spin column. After the spin column was washed twice with low salt wash, the mRNA was eluted with the elution buffer. The mRNA was precipitated and its amount was estimated on an EtBr plate.

Subsequently, cDNA was synthesized using Zap-cDNATM Synthesis Kit from Stratagene:

a. ZAP-cDNA SYNTHESIS:

The mRNA isolated by the Invitrogen kit was added to RNase Block II and linker-primer provided by the kit. The nucleotide mixture added was methylated. After the mixture was left at room temperature for 10 minutes to allow annealing between the mRNA and the primer, MuLV reverse transcriptase was added. The reaction was incubated at 37°C for 1 hour. For the second strand synthesis, the second strand nucleotide mixture, RNaseH, and DNA polymerase I were added and the reaction was incubated at 16°C for 2.5 hours.

The reaction was extracted with phenol:chloroform(1:1). Then precipitation with sodium acetate followed. To pellet, the tubes were centrifuged at 4°C for 60 minutes.

The ends of the cDNA generated were blunt ended by T4 DNA polymerase at 37°C for 30 minutes. EcoRI adaptors were ligated to the ends of the cDNA with T4 DNA ligase overnight at 8°C. The ligase was heat-inactivated at 70°C for 30 minutes. Then, the ends were kinased with T4 polynucleotide kinase for 30 minutes at 37°C. Xho I enzyme was added to delete one EcoRI end and cleave at the Xho I site. The resultant cDNA was phenol:chloroform (1:1) extracted and precipitated and finally resuspend in 10 µl of sterile water.

b. LIGATING cDNA INTO VECTOR ARMS:

2.5 µl of the resuspended cDNA were added to 1.0 µl of Uni-ZAP XR vector (λ vector arms provided by the kit) and ligated together by T4 DNA ligase overnight at 12°C and then for 2 hours at room temperature.

c. PACKAGING THE LIBRARY:

An overnight culture of *Escherichia coli* (PLK-F') was grown, and 1 μ l of the ligation was added to Freeze/Thaw extract. 15 μ l Sonic extract was added to the mixture and the tube was incubated at room temperature for 2 hours. Then 500 μ l λ phage dilution buffer followed by 20 μ l of chloroform were added. The supernatant was then ready for titering.

d. PLATING:

1 μ l of the library was added to 200 μ l of O.D.600=0.5 PLK-F' cells, preincubated at 37°C for 15 minutes with slow shaking, added to 3 mls of top agar (48°C), then plated immediately onto prewarmed NZY agar plates and incubated at 37°C overnight.

e. AMPLIFICATION OF THE LIBRARY:

Aliquots of the packaged lamda clones or amplified suspension containing approximately 50,000 recombinant bacteriophages with 600 μ l of the host cells were plated following the plating procedure mentioned above on 150 mm plates and the incubation time was cut down to 8.5 hours. SM buffer was added to each plate (12ml/plate), and pooled together(120 ml) followed by addition of 0.36 chloroform.

f. LIBRARY SCREENING:

After titering the library to determine the concentration, 70 μ l of the library (50,000 plaques/plate) and 600 μ l of the XL1-blue host were plated on each plate, and incubated at 37°C for 8 hours. The plates were

refrigerated at 4°C for 2 hours, and the plaques were transferred for 2 minutes onto labeled NEN nylon membranes. The membranes were denatured in 1.5 M NaCl, 0.5 M Na OH for 2 minutes and then neutralized in 1.5 M NaCl, 0.5 Tris-HCl, pH 8.0 for 5 minutes. The membranes were dried briefly on Whatman 3MM filter paper and stored until prehybridization in a dry place. Prehybridization, hybridization, washing, and exposure followed similar procedure to that mentioned in the Northern analysis section.

4. Amplification of Cytokine Message from the Pancreas using the PCR :

cDNA was synthesized from pancreatic mRNA of an acutely diabetic NOD mouse (NOD#50) as described above. The primers used for this amplification were synthetic 23-mer oligonucleotides corresponding to bps 144-167 and complementary to bps 567-590 of the published murine TNF α sequence¹¹. 300 ng of each primer were used in each reaction along with 1 μ l of DNA, 3 μ l of 90% buffer, and 10 U of Taq polymerase in a total volume of 30 μ l. The PCR machine (Tyler Research Corp) was programmed 94°C for denaturation, 65°C for annealing, and 72°C for extension. This cycle was repeated for 35 times. The positive control used in this experiment was a pGEM.3Z plasmid containing the same 446 bp fragment mentioned above. The products of this amplification were run on 1% agarose gel along with 1 μ g of genomic λ DNA restricted with HindIII as a molecular weight marker.

In order to estimate roughly the amount of TNF α message in the diabetic pancreas, serial dilutions were made from both the experimental

NOD#50 as well as the positive control for TNF α were made. Another PCR experiment was carried out with these dilutions, and the resultant reactions were run on 1% agarose gel with the λ DNA marker.

III. RESULTS

1. Histological Examination :

Microscopic examination confirmed the presence of insulitis in all acutely diabetic NOD mice. Plates 3.1, 3.2, and 3.3 show the histological examination from NOD mice # 15, 30, and 34 respectively. These pancreata were used later for the Northern analysis. In plates 3.1 and 3.2, the infiltrate is only peripheral and the majority of the islet cells appear to be normal. Plate 3.3 shows the islet of Langerhans from NOD#34 after the infiltrate had completely covered the islet and the majority of the cells were destroyed.

Plates 3.4a, 3.4b, and 3.4c are all taken from the freshly diabetic NOD#50. Plate 3.4a shows a small section of an islet that has not been infiltrated yet, while plate 3.4b shows an islet that is partially infiltrated. Plate 3.4c however, contains an islet that is already destroyed by the infiltrate.

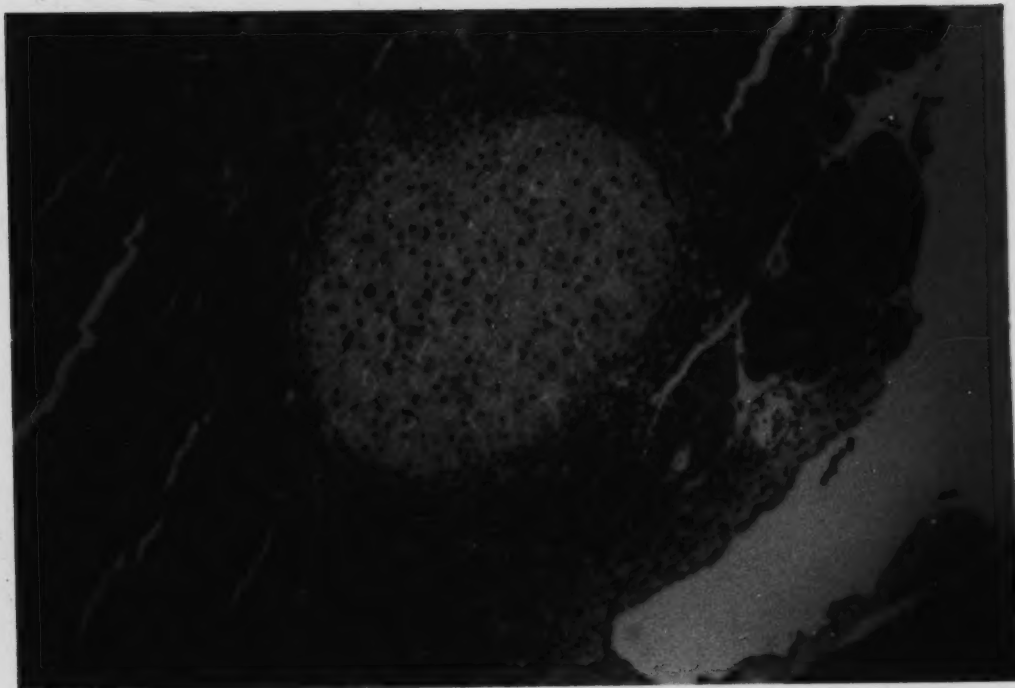


Plate 3.1 : H and E stain showing a section from NOD#15's pancreas and the infiltration of lymphocytes around the islets of Langerhans.



Plate 3.2 : H and E stain showing a section from NOD#30's pancreas and the infiltration of lymphocytes around the islets of Langerhans.



Plate 3.3 : H and E stain showing a section from NOD#34's pancreas and the infiltration of lymphocytes which has completely covered the islets of Langerhans.



Plate 3.4a : H and E stain showing a section from NOD#50's pancreas. No infiltration of lymphocytes around the islets of Langerhans is seen in this section.

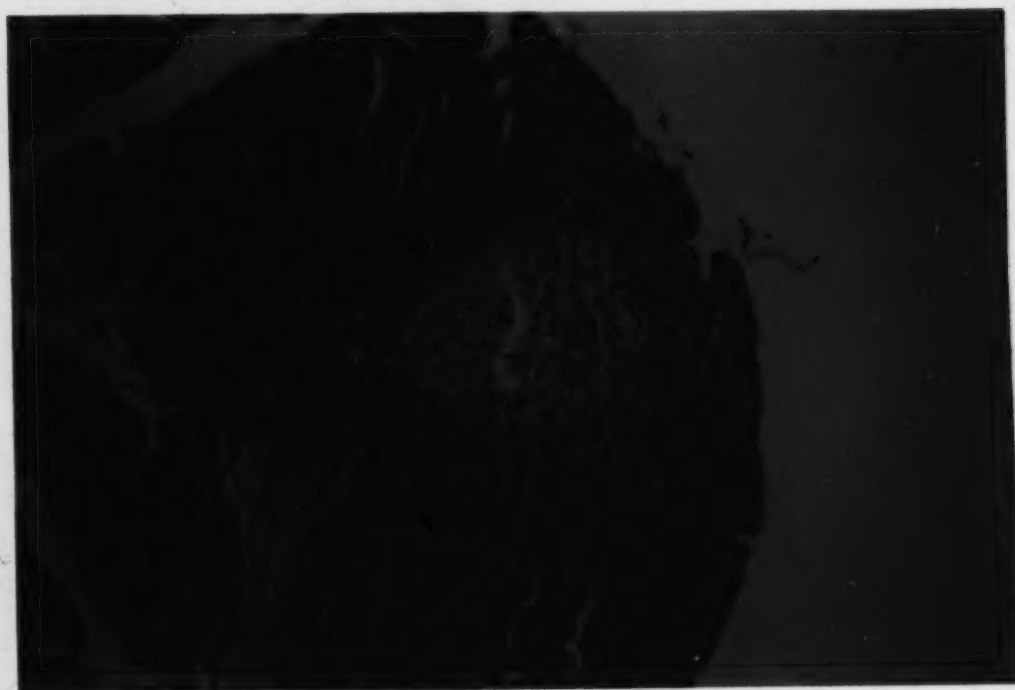


Plate 3.4b : H and E stain showing a section from NOD#50's pancreas. Some infiltration of lymphocytes around the islets of Langerhans is seen in this section.



Plate 3.4c : H and E stain showing a section from NOD#50's pancreas and the infiltration of lymphocytes which has completely covered and destroyed the islets of Langerhans.

2. Northern Analysis :

Total RNA extraction from NOD pancreata #15, 26, 27, 28, 30, 33, and 34 as well as from Balb/c pancreata #1, 2, and 3 was quite successful as determined by the amount of total RNA and by the 260/280 optical density ratios (a ratio of 1.7 or over represents a very pure preparation because it represents the amount of RNA extracted in relation to the amount of protein, including nucleases, present in the preparation). The amount of RNA was determined according to the equation:

$$\text{Amount of RNA} = \text{O.D. reading} \times \text{dilution factor of the sample} \times 40 \mu\text{g /ml}$$

Table 3.1 lists the readings of the optical density of the NOD RNA samples, their concentrations, and their amounts:

Table 3.1:

Total RNA extraction from NOD and Balb/c pancreata:

Sample	O.D. Reading		Ratio 260/280	Amount (mg)
	260nm	280nm		
NOD#15	0.732	0.366	1.99	0.585
NOD#26*	0.775	0.413	1.87	0.650
NOD#27*	1.656	0.857	1.93	1.324
NOD#30	1.052	0.528	1.99	0.841
NOD#31*	1.239	0.620	1.99	0.990
NOD#33	0.450	0.221	2.03	0.360
NOD#34	1.873	1.027	1.82	1.498
Balb/c#1	0.890	0.449	1.98	0.712
Balb/c#2	1.665	0.860	1.93	1.330
Balb/c#3	1.268	0.641	1.97	1.010

* denotes mice that were injected with cycloheximide six hours prior to sacrifice.

In addition, plate 3.5 shows the agarose/formaldehyde gel for these samples. The presence of intact 28s, 18s, as well as 5s ribosomal RNA bands indicates that the RNA extracted was hardly degraded. This was an excellent result achieved since numerous previous RNA extraction from the pancreas always resulted in degraded RNA.

No positive bands corresponding to $\text{TNF}\alpha$ or $\text{IFN}\gamma$ were seen on the autoradiograms after probing the blots from these gels with these probes. Only some nonspecific hybridization to the marker lanes was seen (results not shown). However, when probed with β -actin cDNA, as positive control, a strong band was seen in NOD#15 and a faint one was seen in Balb/c 3 (plate 3.6).

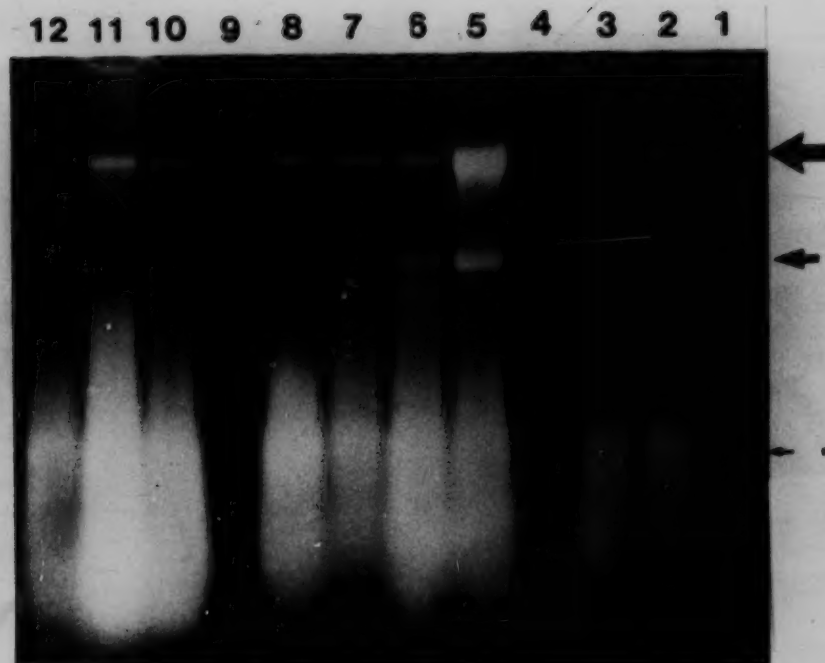


Plate 3.5 : The agarose/formaldehyde gel showing total RNA extracted from NOD and Balb/c mice pancreata (arrows show the 28s, 18s, and 5s bands):

- Lane 1 : Balb/c#1
- Lane 2 : Balb/c#2
- Lane 3 : Balb/c#3
- Lane 4 : RNA 1.77-0.16 kb ladder (marker#1)
- Lane 5 : NOD#15
- Lane 6 : NOD#30
- Lane 7 : NOD#33
- Lane 8 : NOD#34
- Lane 9 : RNA 9.5-0.24 kb ladder(marker#2)
- Lane 10: NOD#26+CHX
- Lane 11: NOD#27+CHX
- Lane 12: NOD#31+CHX



Plate 3.6 : Northern blot analysis of total RNA from the NOD and Balb/c mice, β -actin is the probe used in this experiment and the arrows show the positive band of 2.0 kb :

Lane 1 : NOD#15

Lane 2 : Balb/c#3

The mRNA extraction for the Northern analysis yielded 2.5, and 10 μ g from the diabetic and the cycloheximide-treated mice respectively (plate 3.7). No positive bands were seen corresponding to TNF α or IFN γ in the mRNA samples (result of the negative Northern analysis not shown).



Plate 3.7 : The agarose/formaldehyde gel showing mRNA extracted from NOD pancreas (big arrows show the 28s, 18s, and small ones show the RNA ladder starting at 1.77kb):

- Lane 1 : RNA 1.77-0.16 kb ladder (marker#1)
- Lane 2 : TNF α positive RNA (+ve control#1)
- Lane 3 : NOD total RNA
- Lane 4 : NOD mRNA
- Lane 5 : NOD mRNA from CHX-treated animals
- Lane 6 : Balb/c total RNA
- Lane 7 : IFN γ positive RNA (+ve control#2)
- Lane 8 : RNA 9.5-0.24 kb ladder(marker#2)

3. Construction and Screening of the cDNA Library:

Using the EtBr spotting method, the amount of mRNA extracted from the freshly NOD#50 pancreas was estimated to be 6.4 μ g. The size of total NOD library obtained was 2.45×10^6 pfu/mg of λ arms. After amplification, the size of the library was estimated to be about 7×10^{11} pfu. The twelve filters, containing 4.2×10^6 pfu, screened with TNF α and IFN γ did show any consistent or stable positive clones. Screening the rest of the unamplified library did not seem like a practical idea since that would require 70 filters.

4. Amplification of the Cytokine Message in the Pancreas using PCR:

The results shown in plate 3.8 prove the presence of TNF α message in the diabetic pancreas from the NOD mouse 50. There is an amplified DNA band equal in size to 446 bp and similar to those obtained from the TNF α positive control used in this experiment.

Plate 4.9 shows a more elaborate experiment with serial dilutions of the cDNA from NOD#50 in order to confirm the previous experiment and to examine the lowest amount of cDNA needed to detect the cytokine message. In addition, the serial dilutions can also help in gaining an estimate of the amount of TNF α message present in the cDNA from the diabetic NOD mouse. Table 3.2 shows the amount of DNA and cDNA used as the starting material in each reaction, the amount of TNF α DNA in the positive control (or

estimate in the case of the NOD), and an estimate of their band intensities on the gel.

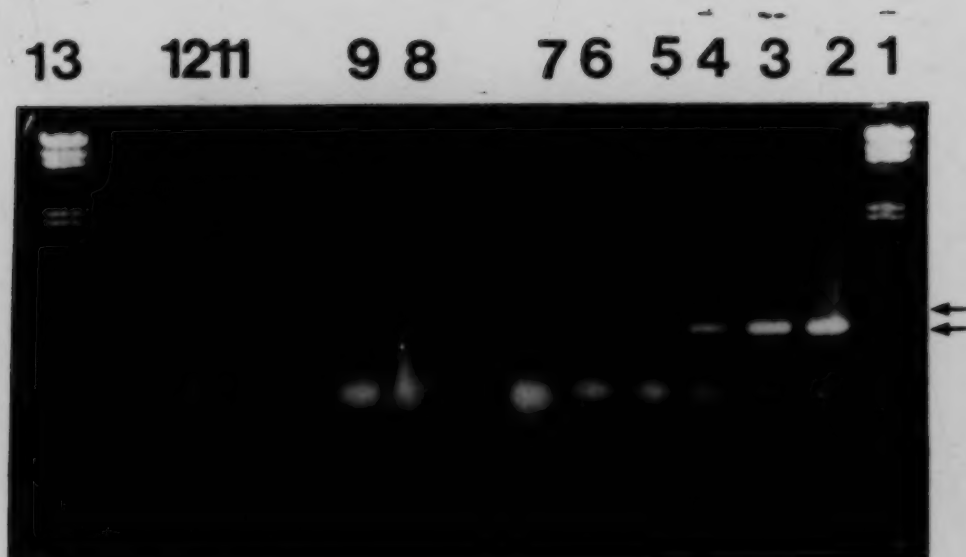


Plate 3.8 : An agarose gel showing the result of the first PCR experiment. Lanes 8 and 9 show a positive band of the expected size (446 bp) of the amplified TNF α (lower arrow), upper arrow indicates the 560 bp marker band :

- Lane 1 : 1 μ g of λ DNA resticted with HindIII
- Lane 2 : TNF α positive control (15 ng of TNF α as starting material)
- Lane 3 : TNF α positive control (1.5 ng of TNF α as starting material)
- Lane 4 : TNF α positive control (0.15 ng of TNF α as starting material)
- Lane 5 : TNF α positive control (15 pg of TNF α as starting material)
- Lane 6 : TNF α positive control (1.5 pg of TNF α as starting material)
- Lane 7 : TNF α positive control (0.15 pg of TNF α as starting material)
- Lane 8 : NOD cDNA (<25 ng as starting material) + TNF α primers
- Lane 9 : NOD cDNA (5 ng as starting material) + TNF α primers
- Lane 10 : NOD cDNA (25 ng as starting material) + IFN γ primers
- Lane 11 : NOD cDNA (5 ng as starting material) + IFN γ primers
- Lane 12 : NOD cDNA (5 ng as starting material) + IFN γ primers
- Lane 13 : 1 μ g of λ DNA resticted with HindIII

15 14 13 12 11 9 8 7 6 5 4 3 2 1



Plate 3.9 : An agarose gel showing the result of the second PCR experiment. The picture shows reactions from serial dilutions from the TNF α positive control and the NOD cDNA. Lanes 9, 10, and 11 show a positive band of the expected size (446 bp) of the amplified TNF α (lower arrow), upper arrow indicates the 560 bp marker band :

- Lane 1 : 1 μ g of λ DNA restricted with HindIII
- Lane 2 : TNF α positive control (15 ng of TNF α as starting material)
- Lane 3 : TNF α positive control (1.5 ng of TNF α as starting material)
- Lane 4 : TNF α positive control (0.15 ng of TNF α as starting material)
- Lane 5 : TNF α positive control (15 pg of TNF α as starting material)
- Lane 6 : TNF α positive control (1.5 pg of TNF α as starting material)
- Lane 7 : TNF α positive control (0.15 pg of TNF α as starting material)
- Lane 8 : 1 μ g of λ DNA restricted with HindIII
- Lane 9 : NOD cDNA (>25 ng as starting material)
- Lane 11: NOD cDNA (5 ng as starting material)
- Lane 12: NOD cDNA (0.5 ng as starting material)
- Lane 13: NOD cDNA (50 pg as starting material)
- Lane 14: NOD cDNA (5 pg as starting material)
- Lane 15: NOD cDNA (0 cDNA as starting material)

Table 3.2:

Plasmid DNA and TNF α DNA amounts and band intensities in the PCR experiment:

Sample	Total amount of DNA (pg)	amount of	Band intensity
		TNF α DNA (pg)	on the gel (U)
		<i>x</i>	<i>y</i>
TNF α positive control	100000	14000	10
	10000	1400	5
	1000	140	2
	100	14	0.5
	10	1.4	0
	1	0.14	0
NODcDNA	<25000	182.2	4
	5000	54.6	3
	500	2.1	0.2
	50	0	0
	5	0	0

The upper half of the table shows the DNA amounts in picograms of the TNF α positive control used in the experiment which is pGEM.3Z plasmid containing a piece of the TNF α gene. The amount of TNF α DNA in each reaction (second column) was calculated as the percentage of the amount of total DNA (i.e. the plasmid and the TNF α insert).

TNF α amount (pg) =

(amount of TNF α sequence)

total amount of DNA (pg) X -----

(amount of TNF α + plasmid sequence)

For example, in row#4 :

446 bp

TNF α amount = 100 pg X -----

3209 bp

The intensity (third column) was estimated visually on a scale from 1 to 10. TNF α amounts from the positive control were plotted against the intensity of the amplified bands from the gel and a logarithmic fit (with $R^2 = 0.802$) was made, using Cricket Graph 1.3 software on Macintosh computers, according to the equation :

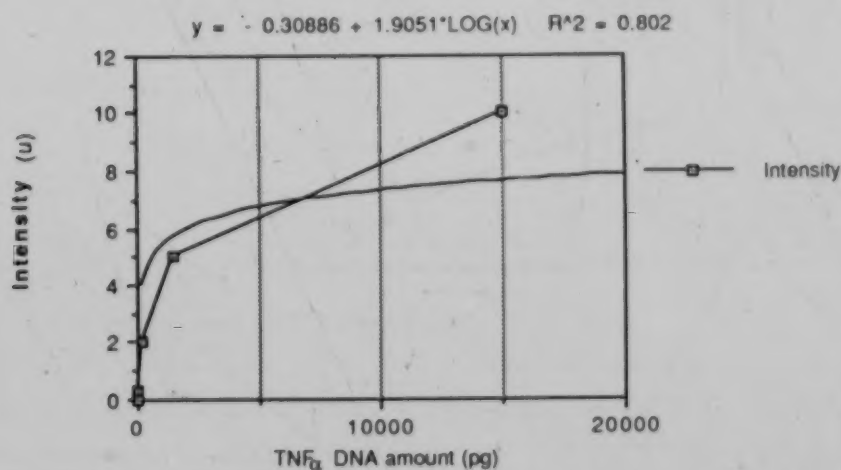


Figure 3.1 : A graph showing the relationship between the starting amount of TNF α DNA in the PCR reactions and the band intensity on the agarose gel.

In order to estimate the amount of TNF α sequence found in each reaction from the pancreatic NOD cDNA, the intensity of the amplified band was also estimated visually on the same scale mentioned above and the value was substituted for y in the equation. TNF α amount (the x value from the equation) was calculated and entered in the lower half of table 3.2 in the third column. Three values were obtained for the three amplified bands seen on the gel.

IV. DISCUSSION

Lymphocytic infiltration of the islets of Langerhans and concomitant and selective destruction of the insulin-producing β -cells are the pathological characteristics of IDDM⁸. This lymphocytic infiltration implicates immunological processes resulting in the destruction of the β -cells. From the vast literature published on IDDM, one can see that there could be more than one mechanism involved. As a result, more than one hypothesis have been postulated. The more recent of them⁸, implicates cytokines released by these infiltrating cells as primarily responsible or at least as participants in the destruction.

Most studies done on the role of cytokines in β -cell destruction so far have been *in vitro* examination of the effect of one or more cytokines on pancreatic β -cells. IL-1¹, IFN γ and IL-6³, as well as IFN γ and TNF α ¹⁰ are cytokines that have been shown to directly inhibit the function and viability of β -cells *in vitro*. However, similar *in vivo* studies were definitely lacking

TNF α gene was shown to be expressed during the development of autoimmune diabetes⁷ using *in situ* hybridization technique. This study, despite the extremely low number of TNF α -producing cells detected (3.7 positive cells/1000 infiltrating mononuclear cells), was the first *in vivo* study to show the presence of TNF α around the islets during diabetes. Furthermore, it is important to remember that this technique is notorious for background problems

In vivo studies of gene expression in diabetes, using Northern analysis or PCR techniques, are not very common. The success of these studies mainly depends on the extraction of undegraded RNA from the pancreas. This is an extremely difficult task since the pancreas is a very ribonuclease rich organ. The ribonucleases found in the pancreas are capable of degrading its RNA within seconds if not treated properly. The conventional CsCl method for extraction of total RNA was not satisfactory, and choosing the effective method was crucial. With the help of the AGPC method⁴, it was finally possible to extract undegraded total RNA from the NOD pancreas.

The results of the Northern analysis for total RNA were negative. This is most likely due the fact that the amount of cytokine message expressed in the pancreas is too little compared to the total amount of message from the pancreas. Generally, the amount of mRNA is about 1% of total RNA. Furthermore, the cytokine message is released from the infiltrating cells which comprise a small number of cells compared to the large number of pancreatic cells. In addition, the pancreas is an active organ that is involved in an on-going process, digestion, and therefore, expected to be a major mRNA, and eventually protein, producer.

Enrichment of the Northern technique by probing the mRNA only and using double the amount of radioactivity did not show any positive bands. The reason could be the enrichment was still not enough. However, when the blot was probed with β -actin to examine the quality of mRNA extracted by the Promega kit, it did not show any positive bands either. Nonetheless, a small positive band was seen in the Balb/c preparation proving the experiment otherwise was successful. These findings have led to the questioning of the quality of the mRNA isolated using the Promega kit.

The Invitrogen kit on the other hand has proven more effective in isolating good mRNA, yet less than expected considering one entire pancreas was used. As seen from table 1, one pancreas yields about 1.5 mg of RNA. Therefore, the amount of RNA expected from such preparation should be about 15 μ g. Nonetheless, this small amount of mRNA was enough for constructing the cDNA library and the PCR amplification experiment.

After the initial screening of the cDNA library, it was obvious that screening for a low abundance message would require intensive labour. In addition, the expense of such a task was too high. Therefore, amplifying the message using the PCR appeared an attractive alternative.

The results obtained from the PCR experiment prove the presence of TNF α in the freshly diabetic NOD pancreas. It was possible to amplify the message from as little as 500 pg of NOD pancreatic cDNA as starting material. A rough estimate of the amount of TNF α message was calculated to be 54.6 pg in the total cDNA from NOD of 500 pg, or 10.9%. This middle value of 54.6 pg appears to be the most reliable value since it lies in the logarithmic (not too steep nor too flat) part of the curve.

TNF α is a cytokine that is known for its cytolytic ability. Its presence in an organ where it is not normally found suggests its involvement in tissue destruction of that organ. In this experiment, TNF α message was found in the pancreas of diabetic NOD mice. On the other hand, it is not found in normal pancreata (previous results from our lab not included in this experiment). Therefore, based on our knowledge of the cytolytic ability of TNF α toward pancreatic islet cells¹⁰, the finding of TNF α message in the pancreas is evidence to support its possible role in directly contributing to β -cell destruction and the development of diabetes.

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Chapter 4

CONCLUSION

I. The results and the hypothesis:

The experiments described in this thesis document the finding of increased TNF α expression in the target tissues of autoimmune diabetes and minor GVHD. These findings provide evidence to support a role for TNF α in directly leading to the immune-mediated tissue injury characteristic of these diseases.

II. TNF α in minor GVHD:

The increased expression of TNF α in the target tissues (liver and tongue) of minor GVHD suggests that the immunological mechanisms that result in this disease are similar to those involved in the pathogenesis of major GVHD. The fact that IFN γ was not detected in this experiment does not completely exclude its involvement in minor GVHD. IFN γ message may have been present at levels undetectable by Northern blot analysis. A more sensitive technique, like PCR, may be able to detect its expression.

III. TNF α in insulin-dependent diabetes:

Many previous *in vitro* studies indicated TNF α and IFN γ may directly destroy the insulin-producing β -cells of the pancreas. These studies were done using rodent model, such as the NOD mouse and the BB rat. The use of these models has been very helpful in studying autoimmune diabetes since they display pathological and clinical characteristics very similar to those found in the human population. Furthermore, it is not yet possible to obtain human tissues in order to study the immunological processes that

lead to diabetes as they mostly occur very early before clinical manifestations.

The cytotoxicity of $\text{TNF}\alpha$ and $\text{IFN}\gamma$, and their synergistic effect, was proven using *in vitro* studies to both mouse⁵, and rat¹¹ islet cells. The source of cytokines may be different in each animal model. In the NOD mouse and human the source of these cytokines is the T-cells from the cytotoxic/suppressor subpopulation². On the other hand, NK cells have been proved to lyse the pancreatic β -cells in the BB rat⁹ and may be the source of the destructive cytokines.

In addition to $\text{TNF}\alpha$ and $\text{IFN}\gamma$, other cytokines were shown to be cytotoxic to the pancreatic β -cells *in vitro*. IL-1 was shown to be cytotoxic to both human and rat islet cells *in vitro*³, whereas IL-6 was cytotoxic to the NOD mouse islet cells⁶. The sources of these cytokines may be different in each animal model. For example, macrophages, which are the source of IL-1, were not seen in the infiltrating cells of the pancreas in human⁷ but they were implicated in the diabetogenesis of the rat^{1,12}.

Our positive finding of $\text{TNF}\alpha$ message in diabetic NOD mouse pancreas is an important piece of evidence for the role of $\text{TNF}\alpha$ in diabetogenesis. In addition, it supports a previous *in vivo* study⁸ which showed the expression of $\text{TNF}\alpha$ in the pancreas of a diabetic NOD pancreas by *in situ* hybridization. However, the number of $\text{TNF}\alpha$ -producing cells was so low that it was difficult to believe it as a true positive finding, especially using this technique which is known for background problems. Therefore, our results support this previous study and show that the amount of message is more than that detected by *in situ* hybridization. In addition, it provides a means for studying the intricate mixture of cytokines in the diabetic

pancreas. It is too early to conclude the absence of IFN γ in the diabetic NOD pancreas from this experiment. Currently, a positive control for IFN γ is being devised and the experiment is being repeated.

IV. Future directions:

The presence of these cytokines in the target tissues of immune-mediated disease is evidence to support their involvement in the development of these diseases. Therefore, it is important to study the type, quantity, and complex interaction of these cytokines at the target site. In addition, it is also important to identify the source producing these cytokines and the cell being targeted. The cellular source of the cytokines could be investigated by combined *in situ* hybridization/immunohistochemistry study; this could likely be a technically daunting task. The target cell susceptibility to direct cytokine-mediated injury by examining the presence of the receptors for these cytokines on the target cells.

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